Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage

Alric López-Martínez*†, David T. Chang*‡, Chin Chiang*‡, Jeffery A. Porter*‡, María A. Ros§, B. Kay Simandl†, Philip A. Beachy‡ and John F. Fallon†

†Department of Anatomy and Neuroscience, University of Wisconsin, Madison, 1300 University Avenue, Madison, Wisconsin 53706, USA. ‡Howard Hughes Medical Institute, Department of Molecular Biology and Genetics, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205, USA. §Departamento de Anatomía y Biología Celular, Universidad de Cantabria, 39011 Santander, Spain.

Background: *Sonic hedgehog* (*Shh*), a vertebrate homolog of the *Drosophila* segment polarity gene *hedgehog* (*hh*), has been implicated in patterning of the developing chick limb. Such a role is suggested by the restricted expression of *Shh* along the posterior limb bud margin, and by the observation that heterologous cells expressing *Shh* have limb-polarizing activity resembling that of cells from the polarizing region of the posterior limb bud margin. It has not been demonstrated, however, that the Sonic hedgehog protein (SHH) alone is sufficient for limb patterning. SHH has been shown to undergo autoproteolytic cleavage *in vitro*, yielding two smaller products. It is of interest, therefore, to determine whether processing of SHH occurs in the developing limb and how such processing influences the function of SHH.

Results: We demonstrate that SHH is proteolytically processed in developing chick limbs. Grafts of cells expressing SHH protein variants that correspond to individual cleavage products demonstrate that the ability to induce patterned gene expression and to impose morphological pattern upon the limb bud is limited to the amino-terminal product (SHH-N) of SHH proteolytic cleavage. We also demonstrate that bacterially synthesized and purified SHH-N, released from implanted beads, is sufficient for limb-patterning activity. Finally, we show that the endogenous amino-terminal cleavage product is tightly localized to the posterior margin of the limb bud.

Conclusions: Our data show that, of the two cleavage products resulting from SHH autoproteolysis, SHH-N expressed in grafted heterologous cells or supplied in purified form is sufficient to impose pattern upon the developing limb. Moreover, the restricted localization of the endogenous amino-terminal SHH cleavage product to the posterior border of the chick limb bud makes it unlikely that its patterning activity results from it being distributed in a broad gradient across the antero-posterior axis. More consistent with the observed localization is a model in which the amino-terminal SHH cleavage product exerts its patterning effects by local induction in or near the polarizing region, initiating a cascade of gene expression that ultimately extends across the developing limb.

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Background

The developing limb bud is an important experimental system for the study of pattern formation [1,2]. As the bud elongates, the antero-posterior axis is organized by mesoderm located along the posterior border of the limb bud — the 'polarizing region' or 'zone of polarizing activity'. The polarizing region is capable of re-specifying limb pattern if grafted to the anterior border of a host limb bud, causing a duplication of digits in mirror image symmetry to the normal antero-posterior sequence [3,4]. This patterning activity is thought to depend upon the release of a signal, possibly a morphogen [5], from the polarizing region. The location of limb-polarizing activity at the posterior margin of the limb bud correlates spatially with the localized transcription of *sonic hedgehog* (*Shh*; also called *vhh-1* or *Hhg-1*), a recently isolated vertebrate homologue of the *Drosophila* segment polarity gene, *hedgehog* (*hh*). Ectopic expression of the *Shh* gene from either the chick or mouse in the anterior limb border results in mirror-image digit duplications that are indistinguishable from those caused by grafts of the polarizing region [6,7]. This suggests that the patterning activity normally present within the polarizing region is encoded by *Shh*.

The secreted products of the *Drosophila hedgehog* gene are found predominantly as two stable protein fragments (HH-N and HH-C), which are derived by internal autoproteolytic cleavage from a larger precursor (HH-U). The cleavage event appears to be crucial for full function [8,9], and the site and probable mechanism of autoproteolytic cleavage is widely conserved among *hh* proteins, including those of vertebrates. Previous studies of *Shh* function in the limb have focused on the consequences of ectopic expression of the *Shh* gene. It is of interest, therefore, to examine the function of SHH in the developing limb at the protein level — to determine whether endogenous *Shh* protein is processed, to determine the relationship of

*The first four authors contributed equally to this work. Correspondence to: John F. Fallon or Philip A. Beachy.

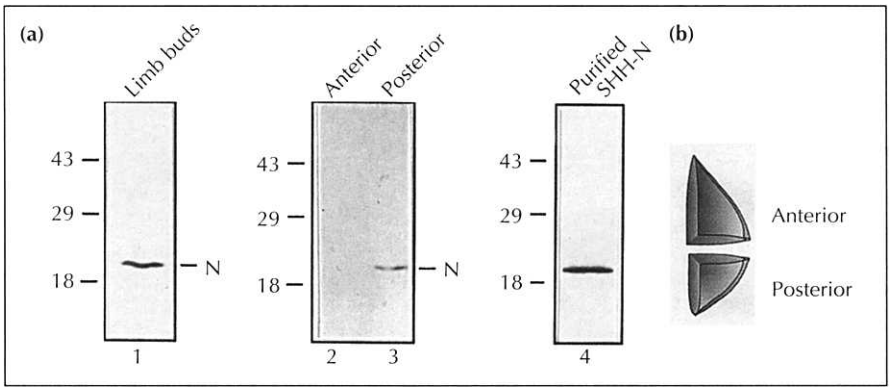

Fig. 1. Endogenous and recombinant *Shh* proteins. **(a)** Antibodies directed against the amino-terminal domain of mouse SHH detect an approximately 20 kD band in immunoblotted extracts from whole limb buds (lane 1) and posterior (lane 3) but not anterior (lane 2) halves of wing buds isolated from embryos at stage 20 and early stage 21. This endogenous protein migrates similarly to SHH-N protein purified from *Escherichia coli* (lane 4). **(b)** The dissection of a limb bud into anterior and posterior halves.

cleavage to patterning activity, and to define the protein species that is active in patterning. The location of the active protein species within the developing limb will also have direct implications for models of limb patterning.

Results

As SHH has been shown to undergo cleavage *in vitro*, in cultured cells and in *Drosophila* embryos [7–9], we tested whether cleavage of endogenous SHH occurs in the developing limb by immunoblotting protein extracts from isolated wing buds. As shown in Figure 1a, antibodies directed against the amino-terminal domain detect an approximately 20 kD protein species; this relative molecular weight corresponds to that reported previously for mouse SHH-N expressed in cultured cells [7], and to that predicted from the location of the conserved autoproteolytic cleavage site within the product of the chick *Shh* gene [7–9]. We therefore confirm endogenous cleavage of SHH.

In order to investigate the roles of cleavage and of the resulting protein fragments in limb patterning, we designed expression constructs (Fig. 2a) that could produce SHH-N in the absence of SHH-C (p*Shh-N*), SHH-C in the absence of SHH-N (p*Shh-C*), or a precursor rendered uncleavable by the replacement of a cysteine with an alanine at the cleavage site (p*Shh-*U_{CA}). These constructs were tested by translation *in vitro* (data not shown) and by immunoblotting of protein extracts from transiently transfected cells of the quail cell line QT6. The immunoblots shown in Figure 2b,c demonstrate that these constructs, expressed in transfected QT6

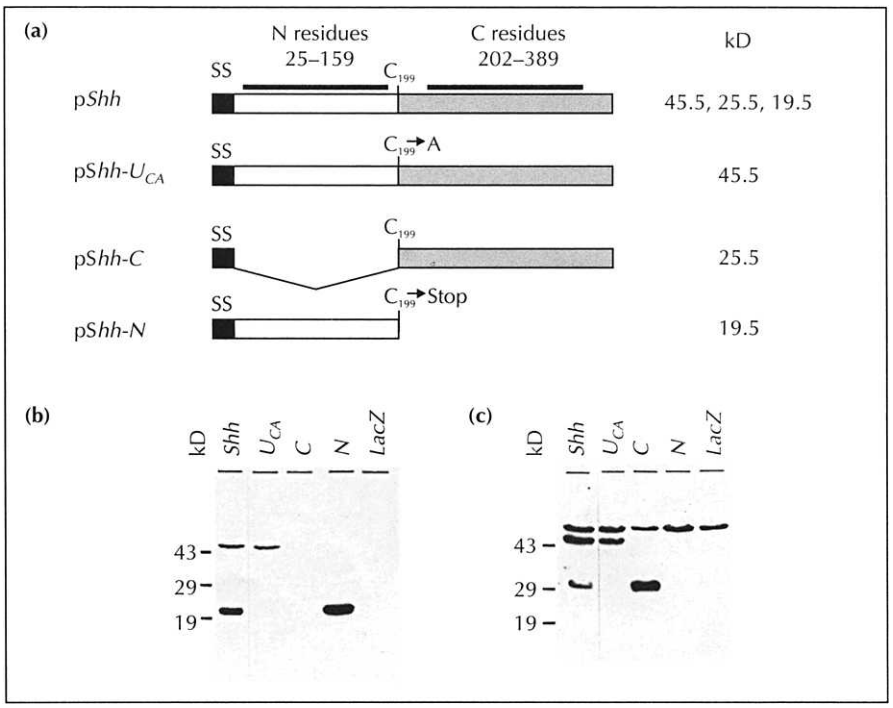

Fig. 2. Expression and processing of SHH in QT6 cells. **(a)** The cDNA-derived inserts into expression constructs that encode mouse wild-type SHH (p*Shh*), an uncleavable precursor (p*Shh-*U_{CA}), and proteins corresponding to the amino (p*Shh*-N) and carboxy (p*Shh*-C) terminal cleavage products of SHH. SS denotes the *Shh* signal sequence, present in all constructs for targeting to the secretory pathway. The expected relative molecular weights of signal-cleaved and autoproteolytically-cleaved (for SHH) protein products are indicated to the right. The black bars in (a) denote sequences used to elicit antibodies specific to epitopes within the amino- (N) and carboxy- (C) terminal domains of SHH. Extracts from QT6 cells transfected with these constructs, and a control p*LacZ* construct, were immunoblotted and detected with antibodies directed against **(b)** SHH-N or **(c)** SHH-C. The uncleaved precursor, which reacts with both antibodies, was detected in cells transfected with the p*Shh* and p*Shh-*U_{CA} constructs. Proteins corresponding to the SHH-N cleavage product were detected in cells transfected with the p*Shh* and p*Shh*-N constructs. As observed previously [7], the SHH-C cleavage product migrates with a mobility corresponding to ~29 kD, somewhat larger than the expected relative weight. Note the absence of lower molecular weight products in QT6 cells transfected with the p*Shh-*U_{CA} construct, indicating a defect in cleavage. The slightly larger cross-reacting species in (c) is not transfection-dependent and provides a control for equality of protein loading.

cells, generate protein species of the appropriate mobilities and antibody reactivities to correspond to the SHH-N, SHH-C and SHH-U_{CA} variants of SHH.

The limb-patterning activities of these constructs and their corresponding protein products were tested by grafting transfected QT6 cells to the anterior border of stage 20 chick wing buds, as was done previously to demonstrate limb-patterning activity of the wild-type mouse *Shh* gene [7]. Whereas QT6 cells transfected with the p*Shh-C* construct had little detectable effect on limb patterning (no duplicated digits), the QT6 cells transfected with the p*Shh-N* construct demonstrated potent patterning activity, with duplications appearing in 100 % of limbs that received anterior grafts; 45 % of these involved a duplicated digit 4 (see Fig. 3 and Table 1). These results compare favorably with results obtained from grafts of cells transfected with p*Shh*, which induced duplications in 86 % of anterior grafts, with 31 % involving a duplicated digit 4 [7]. The p*Shh-*U_{CA} construct had minimal digit-duplication activity (only 2 of 23; see Table 1), and only an additional digit 2 was induced. The SHH-N product therefore appears capable of all the patterning activities of the full-length protein providing it is synthesized as a precisely truncated product, but displays severely attenuated activity when it is part of the SHH-U_{CA} precursor. The latter reduced activity may be due to the interference of SHH-C residues with SHH-N signaling or receptor-binding in the uncleaved precursor; alternatively, appropriate secretion of the precursor may be blocked by the loss of cleavage. We note that comparisons of grafts expressing these constructs can only be qualitative, as protein levels were not precisely controlled.

We also examined, by *in situ* hybridization, the effects of SHH-N and SHH-C on the expression of genes that are proposed to respond to *Shh* signaling. Embryos with anterior wing bud grafts of transfected QT6 cells were processed 6–48 hours after grafting in order to assay for ectopic expression of *hoxd-11* and *hoxd-13*. As shown in the 24 hour time-point in Figure 3, grafts of QT6 cells transfected with p*Shh-N* (Fig. 3b,c), but not those transfected with p*Shh-C* (Fig. 3f,g), induced *hoxd-11* and *hoxd-13* expression in the anterior border, first detected

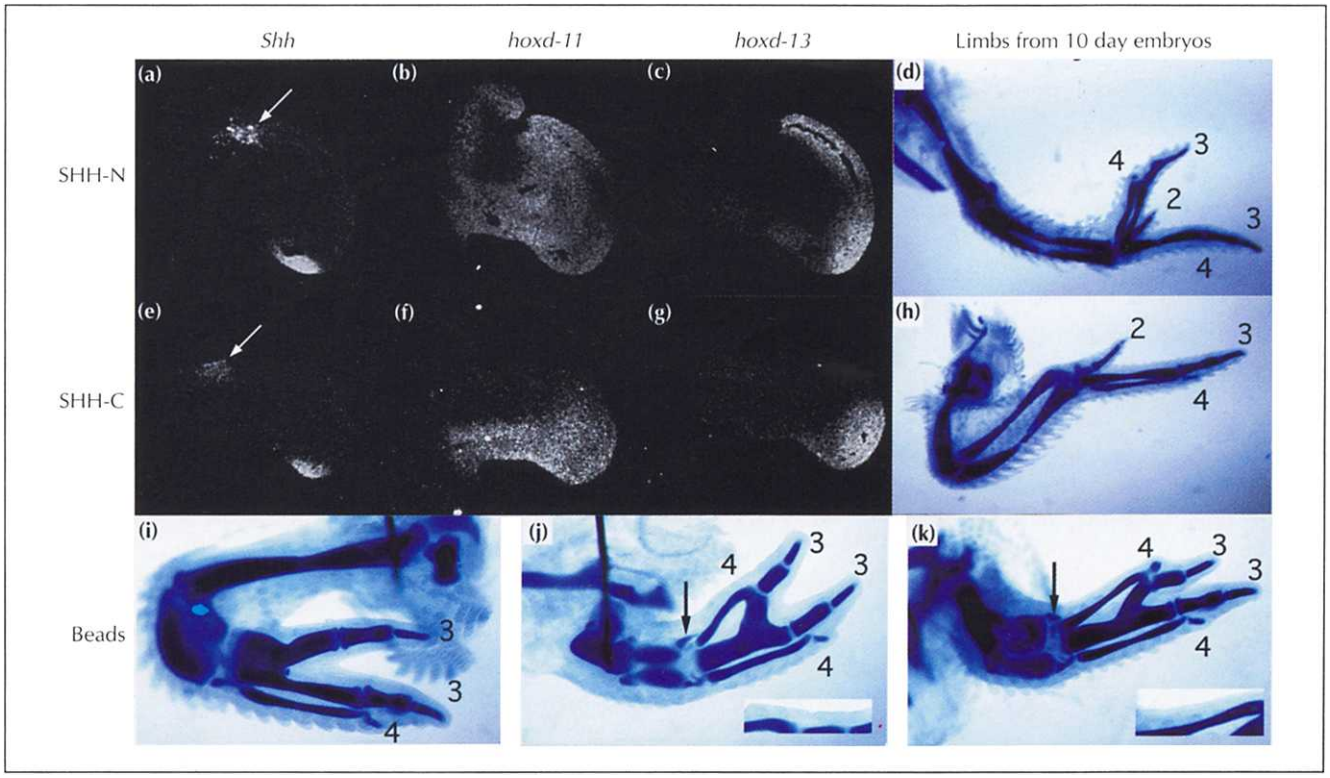

Fig. 3. Effects of SHH-N and SHH-C upon *hoxd* gene expression and limb patterning. QT6 cells transfected with **(a–d)** p*Shh-N* or **(e–h)** p*Shh*-C constructs were grafted to the anterior border of stage 20 chick wing buds. The expression of (a,e) *Shh*, (b,f) *hoxd-11*, and (c,g) *hoxd-13* was detected by *in situ* hybridization 24 h after graft implantation. The digit pattern resulting from grafting QT6 cells transfected with (d) p*Shh-N* (4–3–2–3–4 mirror-image duplication; the duplicated ulnare carpal is out of the focal plane) or (h) p*Shh*-C (2–3–4, the normal digit pattern) is shown seven days after graft implantation. Note that mouse *Shh* mRNA expressed from the transfected p*Shh*-N and p*Shh*-C constructs is detected in the grafted cells, as indicated by white arrows in (a,e). **(i)** Duplication of digit 3 resulting from implantation of a single heparin–agarose bead soaked with purified SHH-N at the anterior border of a stage 20 limb bud. **(j,k)** Mirror-image digit duplications resulting from application at stage 19 of a single heparin-agarose bead soaked with purified SHH-N (digit pattern 4–3–3–4, see Table 1). Other posterior structures induced in the anterior border were the ulnare carpal (black arrow) and feather germs (see insets). Note that the humerus, radius and ulna in these bead-implant experiments were consistently shortened and malformed, in a similar fashion to the short, thick ulna formed after proximal expression of *Shh* is induced by fibroblast growth factor-4 (FGF-4), released from a posterior-proximally placed bead [27].

Table 1. Skeletal element duplications induced by either grafts of QT6 cells transfected with *Shh* expression vectors or beads soaked in purified SHH-N.

			% with most posterior duplicated digit (*n*):				
	Graft (*n*)	**Extra* (*n*)**	**2**	**3**	**4**	**Duplicated ulnare (*n*)**	**Normal (*n*)**
Transfected QT6 cells	p*Shh-C* (32)	6% (2)	0	0	0	0	94% (30)
	p*Shh-N* (33)	0	6% (2)	49% (16)	45% (15)	30% (10)	0
	p*Shh-*U_{CA} (23)	13% (3)	9% (2)	0	0	0	78% (18)
Beads	Control beads (7)	0	0	0	0	0	100% (7)
	SHH-N beads (22)	0	0	77% (17)	14% (3)	30% (6)	9% (2)

*Extra cartilage not identifiable as a digit.

16 hours after grafting (data not shown). The pattern and kinetics of *hoxd* gene induction by ectopic expression of SHH-N are similar to those induced by polarizing-region grafts or by ectopic expression of wild-type *Shh* [10,11]. By contrast, *hoxd-11* and *hoxd-13* expression was unaffected by ectopic expression of SHH-C.

Although the QT6 cell line used in the grafting experiments does not on its own influence limb patterning [7], the possibility remains that SHH-N induces the production of the active patterning factor by QT6 cells or, alternatively, that QT6 cells produce a co-factor necessary for the patterning activity of SHH-N. To address these possibilities, SHH-N was purified from a bacterial source (Fig. 1a), adsorbed onto Affi-gel Blue or heparin beads, and grafted to the anterior border of stage 19–21 wing buds. As shown in Figure 3 and summarized in Table 1, digit duplications were induced by placement of a single SHH-N-loaded bead in 91 % of the cases, with most of these duplications consisting of an additional digit 3. In three specimens, mirror-image duplications developed that consisted of digits 4 and 3, the ulnare carpal and posterior feather germs. In contrast, anterior grafts of beads soaked in control bacterial extracts did not result in digit duplications or induction of posterior structures. As previous reports suggest that the degree of polarizing activity correlates directly with the number of polarizing-region cells implanted in the anterior border [12], we presume that the relatively low number of digit 4 duplications induced by SHH-N released from a bead is due to insufficient levels of SHH-N released over time. This may be due to instability of SHH-N, or because the kinetics or spatial characteristics of SHH-N release are not sufficiently similar to those of cell grafts. These results show that a localized source of purified SHH-N can exert a posterior patterning influence on the developing limb, which, in the best of cases, is comparable to the effect of a polarizing-region graft.

Having established that SHH-N is the product of SHH autoproteolysis that is active in limb patterning, we examined the localization of the amino-terminal cleavage product within developing limb buds. Immunoblotting of protein extracts from limb buds (dissected as in Fig. 1b) with an antibody directed against the amino-terminal domain demonstrates that SHH-N is restricted to the posterior half of the developing limb (Fig. 1a); similar results were recently reported by Bumcrot *et al.* [13]. The resolution of this analysis was extended by whole-mount immunohistochemistry, which revealed the predominant localization of SHH-N to be a region at the posterior margin of the limb, close to, if not coincident with, the polarizing region (Fig. 4a,b). We cannot rule out the possibility that SHH-N is present at more anterior locations within the mesoderm, but both the immunoblots and the whole-mount immunohistochemistry indicate that this is not at a high level. Consistent with there being a highly effective mechanism for limiting diffusion, SHH-N protein synthesized by QT6 cells in the anterior margin is retained near the graft site (Fig. 4c); this may be due to interaction with the extracellular matrix. We note that the mechanism of SHH-N retention near the graft site is distinct from that involved in maintaining the

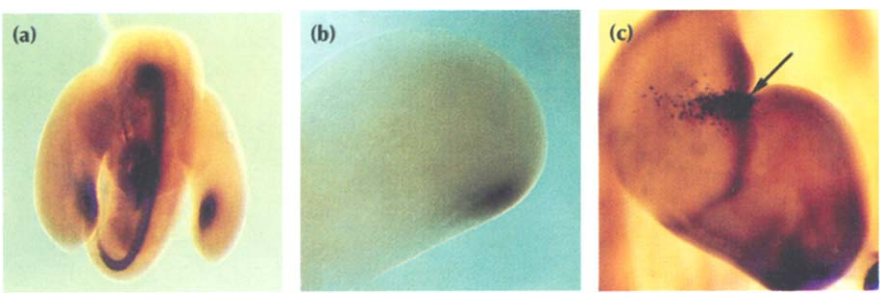

Fig. 4. Immunolocalization of SHH within the developing limb bud. Antibodies directed against the amino-terminal domain of SHH were used for immunolocalization studies in the embryonic chick. **(a)** A whole embryo (stage 26), showing prominent staining in the floor plate, notochord and posterior border of the limb bud, with no immunoreactivity detected in the anterior part of the limb bud. **(b)** Higher magnification view of a limb bud (stage 22), showing tight localization to the posterior border. **(c)** Immunolocalization of SHH in a whole limb bud 24 h after receiving a graft at its anterior border of QT6 cells transfected with the p*Shh*-N construct. Note that the immunoreactivity remains close to the site of the graft, at the anterior border (arrow).

amino-terminal cleavage product tightly associated with the cell in culture, which appears to require cleavage from an intact precursor ([9]; data not shown).

Discussion

Our results indicate that SHH in the limb bud is present in a proteolytically cleaved form, and further suggest that cleavage of SHH is necessary for full patterning activity. The amino-terminal product of this cleavage induces the expession of 5′ genes within the *hoxd* cluster and can reprogram limb pattern when expressed ectopically from heterologous cell grafts at the anterior margin of the limb bud. Furthermore, the patterning activity of purified protein when supplied from a localized source on the anterior margin rules out the possibility of a requirement for other factors expressed from these heterologous cells, and argues that SHH-N alone is sufficient for patterning activity. We note that ectopic expression of HH-N alone in *Drosophila* produced phenotypes identical to those reported for ectopic expression of wild-type HH [9]. SHH-N has also been shown in tissue culture to be sufficient for the induction of sclerotomal markers in presomitic mesoderm [14], and for the induction of floor plate and motor neuron fates in the developing neural tube [15,16].

The observation that *hoxd* gene induction along the anterior margin occurs within 16 hours of exposure to SHH-N is reminiscent of previous reports that signaling from a polarizing-region graft at the anterior limb bud border is required only for approximately 15–20 hours in order to induce a stable response that results in a duplication [17,18]. These observations suggest that pattern specification by the polarizing region can occur within a time period that is less than the total time required for elaboration of the patterned elements, and need not therefore be a continuous process throughout limb development (see [19]).

As previous models of signaling by the polarizing region have proposed, we observe a high concentration of endogenous SHH-N in the posterior border of the limb bud. This distribution, however, appears to be discrete, as we have failed to detect anterior localization of the amino-terminal domain. The form of SHH active in signaling thus appears not to be distributed in the broad gradient that would be predicted by morphogen models of limb patterning. We cannot, however, rule out an anterior localization of endogenous SHH-N at levels too low for detection by either of the two techniques used.

Even if the amino-terminal cleavage product does not itself function directly as a morphogen, it might induce expression of another molecule which does function as a morphogen and which is localized in a broad gradient across the limb bud. As an alternative, a consecutive series of domains of gene expression might be initiated from the posterior margin of the limb bud. We note that localized expression of fibroblast growth factor-4 (FGF-4) in the posterior apical ectodermal ridge, and of bone morphogenetic protein-2 (BMP-2) in the mesoderm, is induced by localized expression of *Shh* [11,20], suggesting that both tissue types have a capacity to respond to SHH signaling. Curiously, whereas BMP-2 transcripts seem to be found in all or most mesodermal cells that contact the domain of amino-terminal SHH cleavage product localization, FGF-4 is expressed in the posterior apical ectodermal ridge but not other ectodermal cells near the *Shh* domain. As neither BMP-2 nor FGF-4 on their own appear sufficient to impose antero-posterior pattern upon the limb [11,20], it would seem that additional signaling molecules, with activities induced by SHH, are likely to be involved in antero-posterior limb patterning. These might act independently of, or collaboratively with, localized signaling molecules that have already been described.

Conclusions

Two cleavage products result from SHH autoproteolysis. We have shown that the amino-terminal fragment, SHH-N, whether expressed in grafted heterologous cells or supplied in purified form, is sufficient to impose pattern on the developing chick limb. Moreover, the endogenous amino-terminal cleavage product of SHH is restricted to the posterior border of the limb bud; its patterning activity is therefore unlikely to result from distribution in a broad gradient across the antero-posterior axis. In keeping with observed localization, we favor a model in which the amino-terminal cleavage product exerts its patterning effects by local induction in or near the polarizing region, initiating a cascade of gene expression that ultimately extends across the developing limb.

Materials and methods

Immunoblotting and purification of SHH-N

Embryos, whole limb buds, and dissected limb buds at stages 20–21 [21] were collected in homogenization buffer (PBS, 1 $\mu g\ ml^{-1}$ leupeptin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 U ml^{-1} aprotinin) at 0 °C and sonicated with three short bursts of 3 W in a Branson cell-disruptor sonifier. Total protein concentrations were determined with the BCA assay (Pierce) following manufacturers recommendations. To enrich for SHH, equivalent total-protein quantities from whole wing bud, and anterior and posterior wing bud preparations, were extracted with Triton-X114 [22], batch-adsorbed to heparin–agarose beads and washed extensively in PBS. Following elution with SDS sample buffer, immunoblotting with affinity-purified antibodies raised against the amino-terminal domain (prepared as in [7]) was carried out with detection by chemiluminescence (ECL, Amersham) using a horseradish-peroxidase-conjugated secondary antibody. The mouse anti-SHH-C antiserum was not effective in detecting chicken SHH-C (data not shown).

Bacterially expressed SHH-N protein was made using the expression vector pGEX2T (Pharmacia) containing *Shh* codons 25–199 followed by a chain-termination codon and fused to GST-coding sequences and a thrombin cleavage site present within the vector. Fusion protein from cleared lysates was purified using

glutathione–agarose beads (Sigma), released from beads by thrombin cleavage, treated to remove thrombin by batch adsorption to anti-thrombin–agarose, and further purified by adsorption to heparin agarose, followed by washing and elution with PBS containing 650 mM NaCl. No protein species other than the SHH-N product could be detected by Coomassie staining of heavily loaded SDS–polyacrylamide gels, indicating a purity of greater than 95 %.

Cell culture, transfection and grafting

Grafts of transfected QT6 cells provide an efficient delivery method for the assay of gene products with potential limb patterning activity. Isolation and characterization of the avian QT6 cell line has been described [23]. QT6 cells were cultured on uncoated plastic culture dishes (Falcon) in growth medium (M199 medium plus Earle's balanced salt solution (Gibco) supplemented with 10 % tryptose phosphate broth, 5 % fetal calf serum, 1 % dimethylsulfoxide, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) with 5 % CO_2.

QT6 cells were transiently transfected by a modified calcium phosphate method [24]. Briefly, after preincubation in transfection medium (DMEM plus 5 % fetal calf serum and 1 % DMSO) 20–25 μg precipitated DNA was added to 70–80 % confluent QT6 cells. After overnight incubation, DNA precipitate was removed and complete growth medium added. Transfected expression constructs were all made with the pCIS vector which includes the cytomegalovirus promoter. These expression constructs carried *lacZ* coding sequences (p*LacZ*); the wild-type *Shh* coding sequence (p*Shh*), *Shh* coding sequences with a Cys 199→Ala mutation that disrupts cleavage (p*Shh-*U_{CA}) [9]; residues 1–198 of *Shh* followed by a chain termination codon (p*Shh-N*); or *Shh* with codons for residues 25–198 deleted (p*Shh-C*). The peptide bond between Gly198 and Cys199 is cleaved in SHH autoproteolysis [9]. Transfection efficiencies were routinely assessed by parallel transfections of plates with equimolar amounts of the p*LacZ* construct.

For grafting, transiently transfected QT6 cells were scraped from tissue culture plates with a Teflon scraper (Falcon) and dissociated by repeated pipetting. Poly-*D*-lysine (Sigma, P1149) was added to the cell suspension to a concentration of 33 μg ml^{-1}. Cells were then pelleted by centrifugation at 103 rpm on a benchtop microfuge for 10 sec. Wedge-shaped fragments were excised from the pelleted cells and grafted into anterior slits made with fine forceps in stage 20–21 chick wing buds.

In situ *hybridization and immunohistochemistry*

In situ hybridization for *Shh*, *hoxd-11* and *hoxd-13* was performed as described previously [25]. The *Shh* probe was derived from a complete chicken *Shh* cDNA. Embryos with anterior QT6 cell grafts were collected at 6, 16, 24 and 48 h and assayed for ectopic *hoxd-11* and *hoxd-13* expression. Sense-strand controls resulted in a hybridization signal at, or lower, than background. Whole-mount immunohistochemistry was performed as described [26], but with extended incubation times with antibodies (3–4 days). Embryos were fixed in either Bouin's solution (Sigma), or 10 % acetic acid and 4 % paraformaldehyde in PBS. See Immunoblotting section for details of antibody preparation.

Bead preparation and grafting

Affi-gel Blue or heparin–agarose beads (Biorad; 200 μm diameter, were incubated either in control bacterial extract or in purified SHH-N solutions for 1 h at 37 °C. Fertile White Leghorn eggs were incubated at 38 °C for 3 days [21]. Beads were placed in the limb bud at stages 19–21 under the ridge at the most antero-distal position.

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